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Procedures for Use With Examination of Stretched and Unstretched Cardiac Non-Myocytes Project

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Whittle Scholar Senior Project

Presented by

Robin F. Thieme

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Dr. Judy Cezeaux

Dr. Bill Jacobs

Kristye Spence

Project Description

This project will examine the effects of mechanical stretching on cultured non-myocyte cells of neonatal rats in an effort to duplicate in vitro the cellular response to high blood pressure in the heart. It is believed that mechanical stretch of non-myocytes will stimulate the renin-angiotensin system and lead to cardiac hypertrophy. This study investigates the change in renin and actin mRNA levels in response to a 10% mechanical stretch.

In order to examine this change, various procedures must be performed on the cells. In addition to culturing and stretching the cells, the mRNA from the cells must be extracted, the mRNA must be transformed into cDNA, the cDNA must be amplified by the Polymerase Chain Reaction (PCR) and interpreted by Gel Electrophoresis. These procedures are what I will be performing and perfecting in an effort to get the best results for the primary investigator of the project.

This project represents a small part of an ongoing study being performed by the graduate students working in The Cellular Biomechanics Laboratory of Dr. Judy L. Cezeaux in the Department of Mechanical and Aerospace Engineering at The University of Tennessee Knoxville. This report gives a summary of the work performed by me during the summer and fall of 1995 while working for Dr. Cezeaux. The aforementioned procedures were all performed in the laboratories of Dr. William R. Jacobs, in the Department of Pediatrics at The University of Tennessee Medical Center. Dr. Jacobs is a collaborator on this project.

The intent of this project is to develop and present protocols for the RNA extraction, the Reverse Transcription (RT), the Polymerase Chain Reaction (PCR), and Gel Electrophoresis procedures. A general background of the project, theory behind the procedures and the protocols for the procedures will be presented here.

Background Information

Cardiac hypertrophy is the most common cause of heart failure in the United States. Most often, this is caused by an increase in the size (width and length) and number of the cardiac cells found in the left ventricle. This is believed to occur as a response to the renin-angiotensin system. This investigation examines the relationship between mechanical stretch and the renin-angiotensin system in myocytes and non-myocytes (Willoughby, 1994). The investigators are specifically evaluating the hypothesis that mechanical loading leads to an increase in renin message by the cardiac non-myocytes.

In order to test this hypothesis, a mechanical stretching device simulating ventricular pressure is used to apply a static uniaxial stretch of 10% for 24 hours to cultured cardiac non-myocyte cells. The cells are isolated from a single litter of neonatal Sprague-Dawley rats and seeded onto two fibronectin coated silicone membranes. Two membranes are seeded so that one membrane may be used as the control (unstretched) for the experiment. The surgical , cell culture and stretching procedures will not be described in this report.

This project will describe the procedures performed once the cells have completed the 24 hour stretch time. The following sections will describe the RNA extraction, RT, PCR, and Gel-Electrophoresis procedures and give a brief background description of each.

Procedures

The first step in finding the amount of renin production from the cardiac cells involves isolating the RNA from the cells. Successful cDNA synthesis begins with the isolation of high quality RNA.

The RNA extraction method used in this investigation is the guanidium isothiocyanate/acid-phenol (GITS) lysate method which yields undegraded RNA from cultured cells by use of a chloroform/phenol extraction procedure. This method is described by Chomczynski and Sacchi (1987). To confirm that the samples were primarily nucleic acids, rather than proteins or organics, the 260/280 nm absorption ratio from optical density was found. The ratio was acceptable at values of 1.7 and greater.

The Polymerase Chain Reaction can only be used to amplify DNA. Therefore, in order to utilize this method, the isolated mRNA must be converted to cDNA by the reverse transcription reaction. This method utilizes an enzyme from a retrovirus and makes a double stranded DNA copy (cDNA) from a single-stranded RNA template molecule.

Once the mRNA extracted from the cells seeded on the membrane has been converted to cDNA, it is ready to be amplified by PCR. PCR is a relatively new procedure for cloning DNA. It can amplify specific regions of DNA by multiple cycles of DNA polymerization. This procedure allows for rapid cloning of small quantities of DNA without use of the living cell. Basically, the 2-stranded DNA double helix is separated, the 2 primer DNA oligonucleotides hybridize to form complementary sequences in the gDNA (or two new DNA strands are formed from the one DNA strand), and the procedure is repeated through several cycles (usually 20 to 30). Each cycle

doubles the amount of DNA from the previous cycle, thus amplifying the DNA. A single cycle requires around 5 minutes and with the automation of the thermal cycler, the fragmented DNA can be amplified in a few hours.

Once the DNA has been amplified, it can be viewed by utilizing the gel electrophoresis method. Each DNA molecule carries a negative charge. By running the DNA through a charged polyacrylamide gel, the various sizes of DNA can be separated at different intervals. The gel can then be stained with ethidium bromide which is absorbed by the DNA. When viewed under ultraviolet light, the stained DNA glows indicating its location. By comparison to the known sizes of the ladder run with the gel samples, the size of the amplified DNA is then detected. In order to know if the product amplified is indeed the desired product, prior knowledge of the size of the nucleotides must be obtained.

The step-by-step procedures utilized in this investigation follow. These are the general procedures used and at times have been altered for improvement.

RNA EXTRACTION VIA GITS PROCEDURE

Once cells are ready on plates, membranes, etc. the following steps should be followed to extract the RNA.

1. PREPARE FOR EXTRACTION PROCESS: Before rinsing plates, mix up working solution of the denaturing reagent. Amount needed is dependent on number of plates.

Each plate needs: 7µl ME (2-Mercapethanol)
1ml stock solution (GITS)

When mixing up the working solution (above) prepare required amount for each plate and an additional 1ml for every two plates. (For example, if you have 2 plates to process, the working solution would need 21µl ME and 3ml stock solution). For additional protocol option, please see note at end.

Each plate will make 2 (1.5 ml epindorf tubes). Label these before beginning extraction process.

Also, prepare the following solutions and keep on ice:

Solution	Amount needed per tube (µl)
2M Na-acetate pH=4.0	50
phenol (H_2O sat. not buffered)	500
chloroform/iso-OH (24:1) (i.e. 2.4ml and 100µl)	100

2. RINSE PLATES: Once plates have been rinsed with PBS, add 1ml (with p1000 pipetter) of the working solution to the plate or membrane. Rinse forcefully, pulling solution up and down and applying a strong liquid force to get cells off the plate or membrane and into a solution. For each membrane, transfer 500µl of the cell solution to each of the 1.5ml tube labeled earlier. Keep the remaining working solution for use later.

3. ADD REAGENTS: Add the following reagents in order on ice to each tube:

50µl 2M Na-acetate pH=4.0
500µl phenol (H_2O sat. not buffered)
100µl chloroform/iso-OH (24:1)

4. STORE SAMPLES ON ICE FOR 15 MINUTES. (Go to hospital)

5. CENTRIFUGE SAMPLES @ 10,000g FOR 20 MINUTES. (While tubes are spinning label another set of 1.5 ml tubes).

6. TRANSFER AQUEOUS LAYER: Carefully remove the aqueous phase (top layer) [DO NOT GET INTO BOTTOM PHASE] and place into labeled tube. Add 1ml of isopropanol.

7. PLACE TUBES @ -20°C TO -80°C FOR AT LEAST 30 MINUTES. If possible, it is best if allowed to precipitate overnight.

8. CENTRIFUGE SAMPLES @ 10,000g FOR 20 MINUTES. (Should see a small pellet)

9. REMOVE THE SUPERNATANT AND DISCARD. Resuspend the pellet in 300µl of the working solution. When doing this, combine same plate aliquots to one tube with 300µl working solution to get a concentrated solution of RNA.

10. REPRECIPITATE @ -20°C TO -80°C FOR AT LEAST 30 MINUTES. Can be left overnight or longer if needed.

11. CENTRIFUGE SAMPLES @ 10,000g FOR 15 MINUTES.

12. REMOVE THE SUPERNATANT AND DISCARD. Add 300µl 75% ETOH/DEPC (will need to mix this up 225µl ETOH/ 75µl DEPC per tube) to wash pelleted RNA.

13. CENTRIFUGE SAMPLES @ 10,000g FOR 15 MINUTES.

14. REMOVE THE SUPERNATANT AND DISCARD. Dry pellet by allowing to sit under hood with cap up or in speed vac. Resuspend pellet in 50µl of DEPC H_2O.

15. MEASURE OPTICAL DENSITY AND LABEL TUBE WITH DATE PREPPED AND CONCENTRATION. (see OD protocol).

Note: An alternative method used when question arose whether the cardiac cells were actually being lysed on the membrane involves altering steps 1 and 2. Instead of adding only 1ml of working solution to the membrane and immediately transferring it to the 2 epindorf tubes, add 3ml of the working solution to the membranes. Let the membranes soak face down in the working solution for about 5-10 minutes on ice. Then aliquot 500µl into 6 epindorf tubes and proceed with addition of reagents as in step 3.

MEASURING OPTICAL DENSITY PROTOCOL

Once the RNA extraction completed, it is important to know concentration of RNA in the sample. The following steps should be followed to measure the Optical Density (OD). It is recommended that the first time this protocol is followed someone who knows what to do helps.

1. DILUTE EACH SAMPLE: For each sample, make a dilution:
   1µl sample RNA
   200 µl DEPC H_2O

   This is most easily done in a 5 ml glass test tube. Add a plastic transfer pipette for each sample. Also include one tube containing 1 ml DEPC H_2O to use as the control.

2. TAKE TUBES TO ROOM #501. The spectrophometer is in this lab.

3. SET REFERENCE WITH DEPC H_2O: Add ≈ 200 µl DEPC H_2O into cuvette. Wipe off cuvette sides with Kim wipe. Be very careful cuvette is expensive!

   Note: > will indicate that you need to push a button on the machine.
   "..." will enclose what the machine's screen will read.
   [...] will enclose what you need to do.

Steps to setting reference: >SET REF
"PLEASE WAIT"
"INSERT REF"
[You must insert the cuvette with blue tick mark facing back corner into the hole when this message is on the screen. If you do not get the cuvette into or out of the machine when requested, the program will have to be repeated. The machine beeps at each step and you must pay attention.]
"REMOVE REF"
[Remove cuvette when it says this!]
[Drain the DEPC H_2O from the cuvette]
This series of steps will set the absorption reading to 0.

4. MEASURE OD OF SAMPLE: Add ≈ 200 µl sample into cuvette. Wipe off cuvette sides with Kimwipe.

Steps to measuring sample OD: >SAMPLE
"PLEASE WAIT"
"INSERT SAMPLE"
[Insert cuvette immediately!]
"REMOVE SAMPLE"
[Remove cuvette immediately!]
[Read concentration (μg/μl) from machine and record]

5. CONTINUE MEASURING ADDITIONAL SAMPLES. To measure the Optical Density of other samples, repeat step 4. You do not need to rinse the cuvette between measurements of each sample, just remove and carefully beat out liquid onto a Kimwipe.

6. CLEAN UP YOUR MESS! When finished, rinse the cuvette with DEPC H_2O and return it to its case. Leave the machine as it was when you began.

The concentration read from the display will be used to get a constant concentration when proceeding with the RT protocol. This helps keep the samples equal when running in PCR.

REVERSE TRANSCRIPTION PROCEDURE

Note: Before setting up samples for RT procedure, turn H_2O baths on 70°C and 37°C.

This protocol utilizes the SuperScript™ Preamplification System for First Strand cDNA Synthesis kit from Life Technologies. It is necessary to have the SuperScript kit in order to proceed with the RT method.

From the OD readings you should determine approximately what amount (µl) of total RNA from the samples is needed to begin RT with relatively equal concentrations of RNA in each sample. (1 to 5 µg of total RNA is necessary for following protocol to be optimized)

1. Mix and pulse down briefly each component before use.

2. Add to an epindorf tube:

Total RNA	10µl
DEPC H_2O	4µl
Oligo (dT)	1µl
	15µl ← Always want 15µl vary the amount of DEPC H_2O relative to amount of Total RNA

   Mix together and pulse down briefly.

3. Heat reaction to 70°C for 10 minutes.

4. Place on ice for 1 minute.

5. Pulse down tube and add to each reaction:

10x buffer	2µl
0.1 DTT	2µl
10MM DNTP mix	1µl
SuperScript RT	1µl

   After adding, mix and pulse down briefly.

6. Incubate at Room Temperature for 10 minutes.

7. Incubate at 42°C for 50 minutes.

8. Incubate at 70°C for 15 minutes.

9. Pulse down briefly. Add 1µl RNase H.

10. Incubate at 37°C for 20 minutes.

Samples can then be stored at -20°C or used immediately for PCR.

POLYMERASE CHAIN REACTION (PCR) PROCEDURE

This protocol will give a general example for setting up a PCR experiment. The quantities of sample, and the primers used will vary each time a new set or samples is run. This protocol provides a general idea of how to set up a stretched and unstretched set of samples using actin and renin primers separately.

This procedure utilizes the Perkin-Elmer PCR Kit.

1. Label a new set of .5µl Epindorf tubes. For each sample, you will need a tube for actin and renin. You will also need an actin control tube and a renin control tube.

2. The most efficient method for setting up a PCR experiment is to make a Master Mix of the reagents needed for each sample/primer reaction. The Master Mix quantities are determined by the number of samples for each experiment. For this example we will assume we have an unstretched sample, a stretched sample, and a control (H_2O) for each primer. So, when making the Master Mix we need 3 samples. Always add another sample in when calculating to insure there is enough. Therefore we will calculate for 4 samples for the Actin Master Mix and 4 samples for the Renin Master Mix. Actin and Renin both have two primer sets (AP1, AP2, RP1, RP2).

Mix up Master Mix as follows:

		ACTIN	RENIN
	vol/tube	total (µl)	total (µl)
10x buffer	5µl	20	20
dH_2O	29.5µl	118	118
P1	5µl	20	20
P2	5µl	20	20
amplitaq	.5µl	2	2
$MgCl_2$	5µl	20	20
	50µl		

Note: The amount of primer added to each reaction will vary to dependent on the concentration of the primer stock. Most primers used in this investigation are stored at dilution factors so that 5µl will give necessary concentration.

3. Add between 5-8µl of sample from RT to labeled tubes.

4. Add 50µl of Actin Master Mix to Actin tubes and 50µl Renin Master Mix to Renin tubes.

5. Add 1-2 drops of mineral oil to seal reactions.

6. Place the tubes in the thermal cycler. The machine used in this investigation is found in room 502.

7. Run thermal cycler program #14.

File 14: 94°C → 1 min
61°C → 1 min
72°C → 1 min
0°C → 1 min
35 cycles
File 11: delay program 72°C → 5 min
File 10: soak program 4°C

8. The thermal cycler program runs approximately 3 hours. When the samples are finished they can be stored at -20°C and are now ready to be run on a gel for interpretation.

GEL ELECTROPHORESIS PROCEDURE

Note: Two methods of gel casting have been used in this investigation: one using Mighty Small™ Dual Gel Caster and the other using disposable gel cassettes. It was quickly determined that using the disposable gel cassettes is much easier and more efficient. Therefore, the procedure listed below describes the procedure for casting a gel in a disposable cassette. However, if a cassette is unavailable and the gel caster needs to be used, instructions can be found in the lab notebook for this project.

1. Take disposable cassettes and seal slot along bottom with red tape. Set the cassette up in a tube drying rack so they will be easy to pour. Recipe pours approximately 4-5 gels.

2. In a 15 ml tube, prepare the 5% gel solution.

30% Acryl-Bisacryl	2.5ml	(Ambion Acrylamide/Bis 19:1)
10x TBE	1.5ml	
ddH_2O	11.0ml	
TEMED	10µl	

3. Once you have the cassettes ready and a clean comb for each, add 75µl NH_4SO_4 (1.0ml ddH_2O with 100µl NH_4SO_4) and mix very gently, careful not to add air bubbles. The NH_4SO_4 should be made fresh every 2 weeks.

4. Add the mixture with a Pasteur pipette carefully between the slot in the top of the cassette. Fill to top ridge. Carefully insert comb with notch securing along front of cassette. Make sure gel is propped up while it polymerizes. Pour all gels as quickly as possible.

5. Let gels sit at room temperature until they polymerize. Any leftover gel solution in your tube can be used as an indicator for when the gel is polymerized.

6. Once the gels are polymerized, they can be stored in a ziploc bag with 1x buffer at 4°C for 2-3 weeks. They are also ready to be used for PCR amplification detection.

7. When you are ready to run a gel, remove red tape from gel cassette. Carefully slide out the combs and rinse wells with ddH_2O. If needed, mark bottom well locations with sharpie on outside surface of cassette.

8. Using the Mighty Small™ II Gel Electrophoresis Unit, align the 3rd ridge of the cassette (lower tab at top of cassette should face back) onto the gray rubber seal of the buffer chamber unit and clamp to form tight seal in the chamber. Press down around outside to secure the seal. Approximately 1-2 inches of the cassette will extend at bottom of the unit. Snap the buffer chamber onto bottom tray of gel unit.

Note: If running two gels, repeat same procedure on other side of buffer chamber unit. If only running one gel, clamp a blank cassette onto other side to complete electric circuit.

9. Mix up 500µl of 1x TBE: 50ml 10xTBE
450ml RNase free H_2O

10. Pour the 1x TBE into the back of buffer chamber behind cassette. Watch to insure that gel is sealed. If leaks occur, reseal the cassette to the unit. Fill chamber to top making sure buffer fills wells. Also fill back chamber to the top. Add remaining buffer to bottom tray. Let gel sit in buffer approximately 20 minutes to distribute to room temperature.

11. Prepare samples from PCR by adding 10µl Gel Loading Solution (Sigma). This will add color to sample and work as a marker when running gel. Also need to get a gel running ladder to load with samples. This serves as a base pair location marker when reading the gel. Add 1µl ladder and 10µl of Gel Loading solution.

12. Using long tip pipette tips, add 10µl of each sample to respective wells. Make sure to keep a record of what sample was loaded in each well lane. Do not forget to load the ladder. These are most often loaded either first or last.

13. After loading, put safety lid in place - red electrode over red electrode and black over black. Attach the voltmeter leads by plugging in correctly matching colors (Red on Red, Black on Black). Switch on power and run

the gel at 100 volts about 1.5 hours. Note: Usually it is necessary to run the loading buffer off the gel and then allow gel to run another 30-45 minutes more.

14. When gel run is complete, disassemble unit. Take gel cassette and using a spatula carefully crack the seal to separate the front and back plates. Carefully slide spatula under gel pulling it away from the cassette. Slide gel into a dH_2O bath. Add 10µl of ETBr. This will stain the gel. Note: Be very careful with ETBr - add Clorox before disposing.

15. Let gel stain in ETBr bath about 10-15 minutes. Carefully rinse with dH_2O after staining.

16. Gel can now be viewed under Ultraviolet light for interpretation.

Results

Unfortunately, the past six months have presented many problems to the investigators thus, the amount of results is minimal and questionable. Preliminary data does in fact indicate that this method will amplify actin and renin from the stretched and unstretched cells. At this time we are not able to quantify these results and give any evidence to support or negate our original hypothesis.

The results we have show amplification of the actin and renin products at their known size. However, additional bands are also being amplified. The origination or these bands is unknown.

We have also run into problems with the RNA extraction method that still need to be identified in order to proceed with the data collection for this project.

Term Definitions

Note: All definitions come from Molecular Biology of the Cell.

Actin - Abundant protein that forms actin filaments in all eucaryotic cells.

base pair - two nucleotides in an RNA or DNA molecule that are paired by hydrogen bonds - for example, G with C and A with T or U.

cardiac muscle - specialized form of striated muscle found in heart, consisting of individual heart muscle cells linked together by cell junctions.

complementary DNA (cDNA) - DNA molecule made as a copy of mRNA and therefore lacking the introns that are present in genomic DNA. Used to determine the amino acid sequence of a protein by DNA sequencing or to make the protein in large quantities by cloning followed by expression.

cytoplasm - contents of a cells that are contained within its plasma membrane and outside the nucleus.

denaturation - dramatic change in conformation of a protein or nucleic acid caused by heating or by exposure to chemicals and usually resulting in loss of biological function.

DNA (deoxyribonucleic acid) - polynucleotide formed from covalently linked deoxyribonucleotide units; serves as the carrier of genetic information.

genome - total genetic information carried by a cell or an organism.

genomic DNA (gDNA) - DNA constituting the genome of a cell or an organism. Often used in contrast with cDNA (DNA prepared by reverse transcription from messenger RNA).

hybridization - process whereby two complementary nucleic acid strands form a double helix during an annealing period; a powerful technique for detecting specific nucleotide sequences.

in vitro - term used by biochemists to describe a process taking place in an isolated cell-free extract. Also used by cell biologists to refer to cells growing in culture (*in vitro*), as opposed to in an organism (*in vivo*). (Latin for "in glass")

lysis - rupture of a cell's plasma membrane, leading to the release of cytoplasm and the death of the cell.

messenger RNA (mRNA) - RNA molecule that specifies the amino acid sequence of a protein. Produced by RNA splicing (in eucaryotes)from a larger RNA molecule made by RNA polymerase as a complementary copy of DNA. It is translated into protein in a process catalyzed by ribosomes.

nucleic acid - RNA or DNA; consists of a chain of nucleotides joined together by phosphodisester bonds.

nucleotide - nucleoside with one or more phosphate groups joined in ester linkages to the sugar moiety. DNA and RNA are polymers of nucleotides.

plasmid - small circular DNA molecule that replicates independently of the genome. Used extensively as a vector for DNA cloning.

RNA (ribonucleic acid) - polymer formed from covalent linked ribonucleotide monomers.

transcription (DNA transcription) - copying of one strand of DNA into a complementary RNA sequence by the enzyme RNA polymerase.

vector - in cell biology, an agent (virus or plasmid) used to transmit genetic material to a cell or organism.

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